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Evaluation of a Whole-Genome Amplification Method Based on Adaptor-Ligation PCR of Randomly Sheared Genomic DNA

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High-throughput genetic studies often require large quantities of DNA for a variety of analyses. Developing and assessing a whole-genome amplification method is thus important, especially with the current desire for large-scale genotyping in previously collected samples for which limited DNA is available. The method we have developed, called PRSG, is based on an adaptor-ligation–mediated PCR of randomly sheared genomic DNA. An unbiased representation was evaluated by performing PCR on 2,607 exons of 367 genes, which are randomly distributed throughout the genome, on PRSG products of hundreds of individuals. An infrequent loss (<1%) of the exon sequence on the PRSG products was found. Out of 307 microsatellites on various chromosomes, 258 (84%) were amplified in both the PRSG product and an original DNA, whereas 49 (16%) microsatellites were lost only in the PRSG product. Array CGH analysis of 287 loci for measuring the relative gene copy number demonstrated that a low bias was detected. Moreover, this method was validated on 100–1,000 laser-captured cells from paraffin-embedded tissues. These data show that PRSG can provide a sufficient amount of genomic sequence for a variety of genetic analyses as well as for long-term storage for future work.

INTRODUCTION

The quality and quantity of DNA samples are critical for large-scale genotyping of single-nucleotide polymorphisms (SNPs) or microsatellites from blood DNA samples, and for mutational analysis and a relative copy number estimation in cancer DNA. An important goal is to supply a sufficient amount of genomic sequence from blood for a variety of genetic analyses as well as for long-term storage for future work and archiving of patient samples, especially paraffin-embedded tissues, from which the yield of DNA is limited. Several methods for this purpose have been reported: establishment of Epstein–Barr virus-transformed B-cell lines from whole blood, whole-genome amplification (WGA) by random or degenerate oligonucleotide-primed PCR (PEP- and DOP-PCR) (Telenius et al., 1992; Zhang et al., 1992; Snabes et al., 1994; Cheung and Nelson, 1996), and multiple displacement amplification (MDA)–based WGA (Dean et al., 2002). The virus-mediated method includes a time-consuming process. Improved PEP- and DOP-PCR have been reported to be used in mutational analysis and comparative genomic hybridization (CGH) for screening genomic imbalances on as few as 10 to 100 cultured or microdissected cells (Kuukasjarvi et al., 1997; Dietmaier et al., 1999; Huang et al., 2000). MDA, which has recently been developed, is based on rolling circle amplification by use of Phi 29 DNA polymerase (Dean et al., 2002). Toward practical use of a WGA method in high-throughput genetic studies, each copy number of many kinds of genomic DNA sequences should be retained in the WGA products. However, assessment of WGA is quite difficult because of the high complexity of the human genome.

We show here a new WGA method, termed PRSG (adaptor-ligation PCR of randomly sheared genomic DNA), which we evaluated by exon amplification, genotyping of 307 microsatelites, and array CGH analysis of 287 loci.

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MATERIALS AND METHODS

PRSG for High-Molecular Genomic DNAs

To shear high-molecular DNA, 0.5–1 μg of DNA prepared from whole blood, cultured cells, or frozen tissues was forced through a small hole in a ruby by a syringe pump by use of an instrument for an automated hydrodynamic shearing, HydroShear (GeneMachines, San Carlos, CA) (Thorstenson et al., 1998). Randomly fragmented DNA ranging from 0.5 to 2 kb in size was purified by phenol extraction followed by precipitation in isopropanol with 20 μg glycogen. The pellet was dissolved in 5 or 10 μl of TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). DNA solution (5 μl, ~500 ng) was mixed with 50 μl of BAL31 reaction buffer and incubated at 70°C for 5 min and 30°C for 5 min, then incubated with 1 μl of 1.5 U/μl BAL31 nuclease at 30°C for 1 min. DNA fragments were purified by phenol extraction followed by precipitation in isopropanol with 20 μg glycogen. The pellet was dissolved in 8 μl of TE. For end filling, 1 μl of 10× T4 DNA polymerase reaction buffer was added and incubated at 70°C for 5 min, at 30°C for 5 min, and then incubated with 1 μl of 1 U/μl T4 DNA polymerase at 37°C for 5 min. DNA fragments were purified by phenol extraction followed by isopropanol precipitation, then dissolved in 25 μl of TE. To amplify the DNA fragments by PCR, *Eco*RI–*Not*I–*Bam*HI adaptor (Takara Shuzo, Shiga, Japan) was ligated to the DNA by use of T4 DNA ligase, 1 μl of DNA solution (20 ng), 2 μl of 10× T4 DNA ligase reaction buffer, 1 μl of 10 pmol/μl *Eco*RI–*Not*I–*Bam*HI adaptor, 1 μl 10 mM ATP, 14 μl water, and 1 μl T4 DNA ligase (350 U) were mixed and incubated at 16°C for 12 hr. A 1 μl (1 ng) sample of the adaptor-ligated DNA was amplified by PCR, which was carried out in a total volume of 100 μl: 1 μl of the ligation mixture, 77 μl DNase-free water, 10 μl of 10× PCR reaction buffer (100 mM Tris-HCl, pH 7.5, 500 mM KCl, 30 mM $MgCl_2$), 1 μl of 100 pmol/μl ER-1 primer (5′-GGA ATT CGG CGG CCG CGG ATC C-3′), 10 μl of 2.5 mM dNTP and 1 μl Ex *Taq* DNA polymerase (2.5 U; Takara Shuzo). The first PCR was cycled 15 or 20 times (95°C for 1 min, 72°C for 3 min), followed by incubation at 72°C for 10 min, and then one fifth of the reaction product was subjected to an additional 5 or 10 cycles of amplification. After phenol/chloroform extraction, the aqueous layer was collected and purified twice by isopropanol precipitation. The amplified DNA pellet was dissolved in TE.

PRSG for Laser-Captured Microdissected Samples

Methanol-fixed, paraffin-embedded esophageal carcinoma tissues were used for laser-captured microdissection (LCM) (Emmert-Buck et al., 1996). The cryostat sections (8 μm) were laser-microdissected with a PixCell II LCM system from Arcturus Engineering (Mountain View, CA). For DNA isolation, the transfer film and adherent cells were mixed with 200 μl of lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5% SDS) at room temperature, extracted with phenol/chloroform, and precipitated in isopropanol with 20 μg glycogen. The DNA pellet was dissolved in 10 μl of TE, and then amplified by adaptor-ligation–mediated PCR after end filling as described above.

Genotyping for Microsatellite Polymorphisms

Some 312 microsatellite markers in the human genome-wide screening set version 9 (Research Genetics, Huntsville, AL), consisting of 387 markers placed at an average intermarker distance of 10 cM, were used (Dubovsky et al., 1995). A unilateral primer of each primer set was labeled with ABI dyes, 6-FAM, HEX, or TED. A 10 ng sample of DNA was suspended in a total volume of 16 μl PCR buffer, containing 1.5 mM $MgCl_2$; 8 pmol specific primer pairs, including one labeled with a fluorescent reagent; 200 mM dNTPs; and 0.5 unit of *Taq* polymerase (PE Biosystems, Tokyo, Japan). The reactions were carried out in a thermal cycler for 10 cycles under the conditions of 94°C for 15 sec, 55°C for 15 sec, and 72°C for 30 sec, followed by 25 cycles under the conditions of 89°C for 15 sec, 55°C for 15 sec, and 30 sec at 72°C, with a final extension of 10 min at 72°C. Amplified products were denatured for 2 min at 95°C, mixed with formamide dye, applied with a size standard marker of 500TAMRA (PE Biosystems) to each lane, and run on a GeneScan Polymer in an ABI Prism 310 DNA Sequencer (PE Biosystems). The size of each fragment was determined automatically by use of GeneScan software.

Array CGH Analysis

Gene copy number was assessed by use of a commercial array (GenoSensor Array 300 v1.0; Vysis, Downers Grove, IL) according to the manufacturer's protocol. The array contains 287 bacterial artificial chromosome (BAC) clones corresponding to various chromosome loci that have been reported to be altered in various human cancers (list available from the manufacturer's web site, http://www.vysis.com/). Briefly, DNA samples isolated

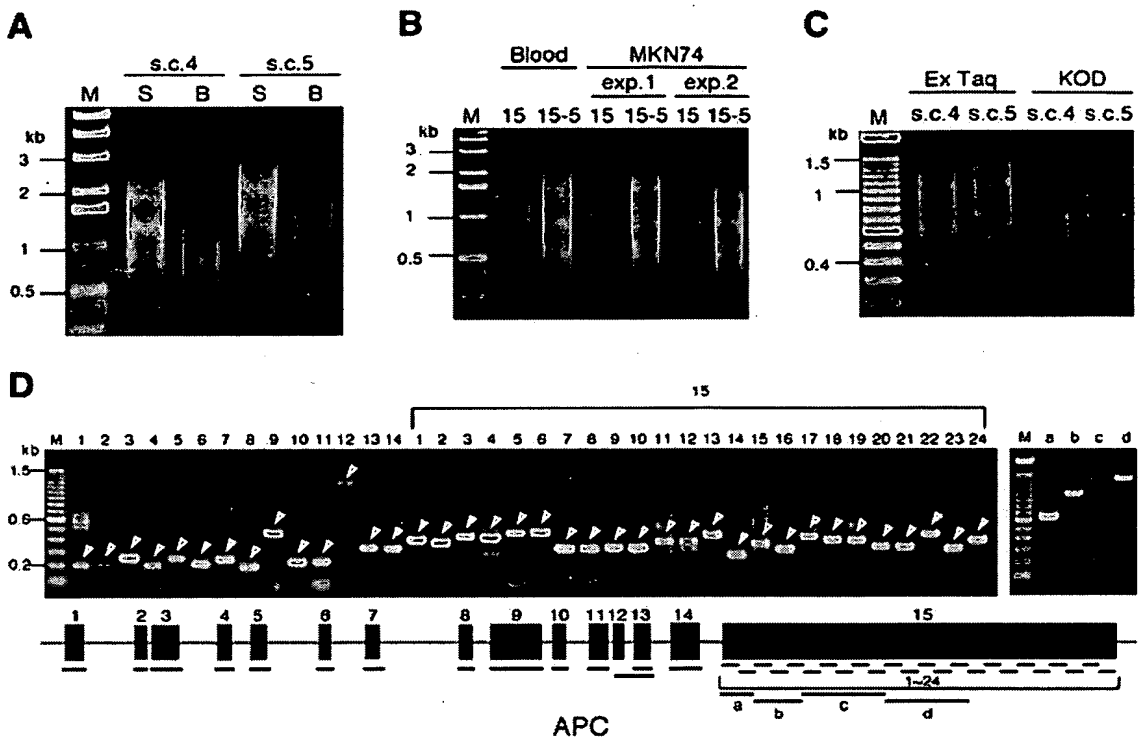

Figure 1. Ethidium bromide–stained agarose gel electrophoretographs of sheared DNAs, DNAs after end filling, PRSG products, and 42 amplified sequences corresponding to all of the 15 *APC* exons from a PRSG product. **A:** One μg of blood DNA (S), sheared at two different flow rates (speed codes [s.c.] 4 and 5) for 20 iterations, and 1 μg of blood DNA fragments (B) after end filling by BAL31 nuclease and T4DNA polymerase. **B:** One μg of blood and MKN 74 gastric cancer cell line DNA, produced by first PCR cycled 15 times (15) and second PCR cycled 5 times (15–5) by use of Ex*Taq* DNA polymerase. **C:** One μg of blood DNA, produced by second PCR cycled 5 times by use of Ex*Taq* polymerase (Ex*Taq*) and KOD-plus DNA polymerase (KOD). **D:** A schematic diagram of the *APC* exons and PCR targets is shown (bottom panel). DNA fragments ranging in size from 0.2 to 1.5 kb, amplified by PCR by use of previously reported specific primers (Groden et al., 1991; Yamakita et al., 1997) from 50 ng of a PRSG product, were analyzed by ethidium bromide–stained 2% agarose gels (top panel). Target DNA fragments for PCR, arrowheads and bars.

from normal human lymphocytes (reference DNA) and tumor samples (test DNA) were labeled by random priming with Cy3- or Cy5-labeled dCTP. The DNA probes (0.1 μg) were mixed with 20 μg of unlabeled Cot-1 DNA and were hybridized to the genomic array, which was then counterstained with DAPI and analyzed by the fluorescent image-capturing system, GenoSensor (Vysis).

DNA Sequencing

Direct sequencing of PCR products corresponding to the exons of any gene obtained from PRSG products and the original genome DNAs was performed by an ABI PRISM 7700 DNA Sequencer (PE Biosystems). Pyrosequencing for genotyping of single-nucleotide polymorphisms was performed by use of the PSQ96 system (Pyrosequencing AB, Uppsala, Sweden).

RESULTS

PRSG

PRSG consists of three steps: step 1, a reproducible hydrodynamic shearing of genome DNA by use of an automated instrument, HydroShear; step 2, adaptor-ligation after end filling by use of BAL31 nuclease and T4 DNA polymerase; step 3, two steps of PCR with low number of cycles. As shown in Figure 1A, most of the sheared DNA fragments fell within a twofold size distribution that is highly reproducible among different flow rates. Although the size of the DNA fragments was slightly shortened by end filling by use of BAL31 nuclease and T4 DNA polymerase (Fig. 1A), the size of the blunt-end DNA fragments was retained even after two steps of PCR (Fig. 1B). From a 1 ng DNA template, PRSG could generate 5 to 10 μg of am-

TABLE 1. Assay of 12 SNPs on PRSG Products from 48 Individuals

SNPs ID	Allelic variation	No. of major homozygous genotypes (G/G)	No. of heterozygous genotypes (G/C)	No. of minor homozygous genotypes (C/C)
IMS-JST102884	G/C	32	15	1
rs140693	C/T	18	29	1
rs175080	G/A	37	11	0
rs357564	A/G	21	20	7
rs462779	C/T	19	24	5
rs689647	C/T	15	26	7
rs1805794	C/G	13	27	8
rs2236405	A/T	38	10	0
rs2237857	G/A	40	6	2
rs2239359	A/G	37	9	2
rs2335052	G/A	21	25	2
rs2602141	A/C	15	26	7

plified products ranging in size from 0.4 to 1.5 kb at speed code 4, or 0.5 to 2.0 kb at speed code 5, when Ex*Taq* DNA polymerase was used (Fig. 1B, C), and 2–5 μg of amplified products from 0.4 to 1.0 kb at speed code 4, or 0.5 to 1.2 kb at speed code 5, when a high-fidelity enzyme, KOD-plus DNA polymerase was used (Fig. 1C).

Retention of Unique Genomic DNAs in the PRSG Products

We first checked for the presence of unique genomic DNA sequences in the PRSG products. An unbiased representation of PRSG was evaluated by performing PCR followed by direct sequencing on 2,607 exons of 367 genes, which were

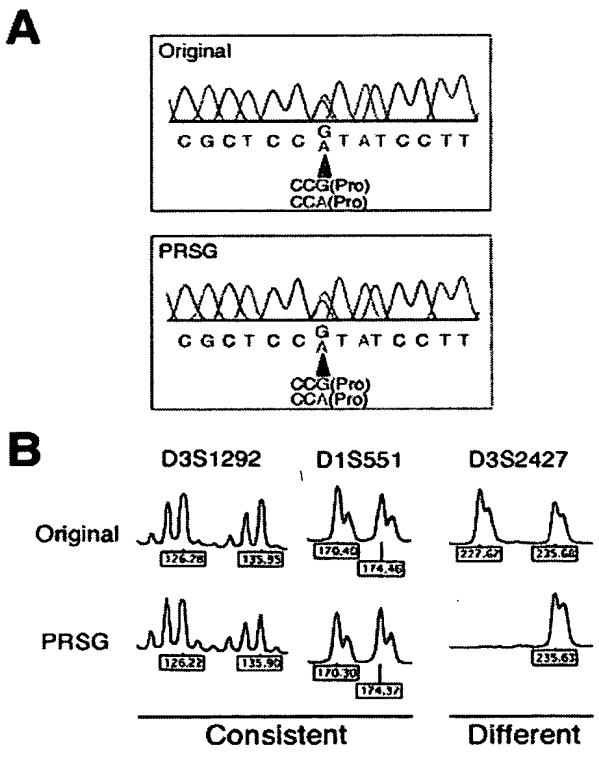

Figure 2. Representative results of SNP detection and amplification of microsatellite markers by use of PRSG products. **A:** The sequence electropherograms showed an SNP in the *c-MET* oncogene, detected in both the PRSG product and a corresponding original genome DNA. The arrowhead indicates the SNP site. **B:** Amplified products were analyzed by an ABI PRISM 310 DNA sequencer and GeneScan software. Three microsatellites (*D3S1292*, *D3S1304*, and *D1S551*) showed a consistent pattern between a PRSG product and the original genome DNA, whereas artificial monoallelic amplification was observed in the *D3S2427* marker.

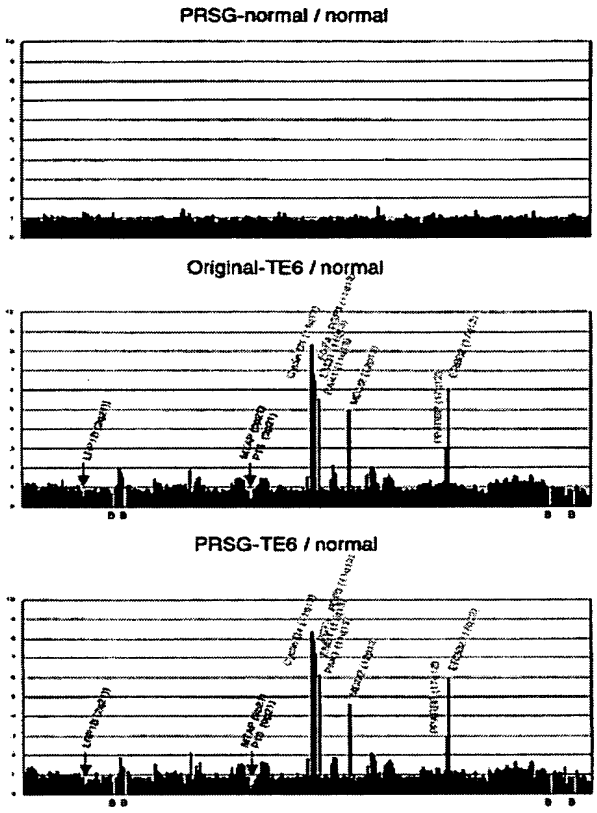

Figure 3. Measuring relative gene copy number by array CGH by use of PRSG products and the original genome DNAs. Fluorescence ratios on all of the 287 chromosome loci between the PRSG products and the original genome DNAs are shown: those between the PRSG-normal male DNA and a corresponding normal male DNA (top panel), between the original TE6 DNA and the normal male DNA (middle panel), and between the PRSG-TE6 DNA and the normal male DNA (bottom panel). Amplified or homozygously deleted gene candidates and their chromosome loci, whose ratios between TE6 DNA and a normal male DNA were changed more than threefold (red) or <0.5-fold (blue), are also indicated. B, no DNA spot on the array used.

randomly distributed throughout the genome, on PRSG products of hundreds of individuals. An infrequent loss (<1%) of the exon sequence in the PRSG products was found (data not shown). The result of PCR for 42 sequences corresponding to all of the 15 *APC* exons is shown as a representative result (Fig. 1D). No loss of any of the 15 *APC* exons in the PRSG products was found. Next, we performed direct sequencing analysis of the PCR products containing exons to examine whether the PRSG products were useful for SNP detection. To verify the conservation of SNPs within the PRSG products, parallel genotyping was performed with both the PRSG products and the corresponding original genome DNAs of 48 individuals. Twelve SNPs with sufficient bi-allelic heterozygosity, out of 11 from database (db)SNP (Sherry et al., 2001) and one from Japanese (J)SNP (Hirakawa et al., 2002), were selected for this comparative examination. The identities, allelic variations, and distributions of genotypes within the population of the selected SNPs are described in Table 1. Genotyping was performed by use of a pyrosequencing method (Alderborn et al., 2000). Identical results were obtained when genotyping the PRSG products and the corresponding original genomic DNAs of 48 individuals (Fig. 2A). These data also suggested that the PRSG products can be applied to large SNP scoring studies by various kinds of methods including Invader, *Taq*Man-PCR, and a detection system by use of a mass spectrometer.

Genotyping for Microsatellite Polymorphisms

Sets of short tandem repeat polymorphisms, called microsatellites, are also useful for loss of heterozygosity (LOH) analysis and for linkage screening of the human genome (Zubenko et al., 1998; Yanagitani et al., 2002), although SNPs are available. Representative data, demonstrating that microsatellites were also retained in the PRSG products, are shown in Figure 2B. In summary, out of 307 microsatellites on various chromosomes, 258 (84%) were amplified in both the PRSG products and the original DNA, whereas 49 (16%) microsatellites were lost only in the PRSG product. More important, 258 microsatelites were amplified repro-

TABLE 2. A High Concordance of the CGH Signals of Changed Genes[a] in TE6

Gene, marker and its locus		Original	PRSG
LRP1B	2q21.2	0.5	0.5
TERC	3q26	2.0	1.9
EIF5A2	3q26.2	1.8	1.5
PIK3CA	3q26.3	1.5	1.4
C84C11/T3	5p tel	1.4	1.6
D6S434[b]	6q16.3	1.1	0.5
D6S311	6q23024	1.9	2.1
EGFR	7p12.3-p12.1	1.5	1.6
MYC	8q24.12-q24.13	1.5	1.5
SHGC-31110	8q tel	1.5	1.6
MTAP	9p21.3	0.3	0.3
CDKN2A(p16), MTAP	9p21	0.4	0.5
TSC1	9q34	1.5	1.7
ABL1	9q34.1	1.4	1.6
H18962	9q tel	1.5	1.6
D11S461	11q12.2	1.6	1.8
CCND1	11q13	8.3	8.4
FGF4, FGF3	11q13	6.9	7.8
EMS1	11q13	6.5	7.2
PAK1	11q13-q14	5.6	6.2
8M16/SP6	12p tel	1.4	1.5
SHGC-5557	12p tel	2.1	1.9
CCND2	12p13	1.7	1.9
MDM2	12q14.3-q15	5.0	4.6
PNN(DRS)	14q13	1.6	1.4
AKT1	14q32.32	2.1	2.1
IGH(D14S308)	14q tel	2.0	1.9
IGH(SHGC-36156)	14q tel	1.8	1.7
FES	15q26.1	1.4	1.6
IGF1R	15q25-q26	1.6	1.8
PACE4C	15q tel	1.4	1.6
PPARBP(PBP)	17q12	3.1	3.0
ERBB2(HER-2)	17q11.2-17q12	6.1	6.0
NCOA3(AIB1)	20q12	1.6	1.7
MYBL2	20q13.1	1.5	1.4
TPD52L2, TOM	20q tel	1.6	1.7
20QTEL 14	20q tel	1.5	1.6
HIRA(TUPLE1)	22q11.21	1.7	1.8
DXYS129	X/Yp tel	0.5	0.5

[a]Genes whose fluorescence ratios between the TE6 DNA (a PRSG product and an original DNA) and a normal male DNA were changed more than 1.5-fold or less than 0.5-fold.
[b]A marker showed an unretained ratio in a PRSG product compared with an original DNA.

ducibly, and 256 out of 258 microsatellites (99%), including 118 out of 122 heterozygous types (98%), showed a consistent pattern between the PRSG product and the original DNA. Although there is a subset of microsatellites that may be lost in the PRSG product, more than 80% of the microsatellites can be useful for a large-scale genotyping.

Measuring Relative Gene Copy Number

In the TE6 esophageal cancer cell line, amplification of *CAB1*, *ERBB2/HER2* (17q12), *CCND1*, *HST1/FGF4*, and *EMS1* (11q13), and homozygous deletion of *P16/CDKN2A* (9p21) were previously reported (Igaki et al., 1995; Akiyama et al., 1997; Ishizuka et al., 2002). To evaluate the locus representation on PRSG, we conducted an array CGH analysis of 287 chromosomal loci by use of the PRSG-TE6 DNA, the original TE6 DNA, a PRSG-normal male DNA, and a normal male DNA. More than 90% of the fluorescence ratios between the PRSG-normal male DNA and a normal male DNA were found within 0.75–1.25 (Fig. 3). This result suggests that the locus representation in the PRSG product is comparable to that on the original genome DNA. The candidates for amplified or deleted chromosomal loci, whose fluorescence ratios between the TE6 DNA (a PRSG product and an original DNA) and a normal male DNA were higher than 1.5 or less than 0.5, are summarized in Table 2. Consistent signals were detected from most of the loci, including the 17q12 and 11q13 amplified loci as well as the 9p21 homozygously deleted locus in both the DNAs compared with a normal male DNA (Fig. 3 and Table 2). These data suggest that PRSG products can be applicable to array CGH analysis for identifying not only amplified but also deleted loci.

Application of PRSG to Paraffin-Embedded Tissues

To amplify a small amount of DNA from old samples by PRSG, LCM on a methanol-fixed, paraffin-embedded esophageal carcinoma showing amplification of the 11q13 locus was performed (Fig. 4A). We were able to amplify 1–10 ng DNA of 100–1,000 cancer cells to hundreds of μg of DNA without the hydrodynamic shearing process, given that DNA fragmentation already occurred in the paraffin-embedded tissue DNA sample (Fig. 4B). On PRSG products from methanol-fixed, paraffin-embedded tissues, when we tested 30 different exons from 140 gastric tumors, all exons from all of the tumors were amplified (data not shown). A result of PCR for 24 sequences corresponding to exon 15 of *APC* is shown as a representative result (Fig. 4C). No loss of the exon sequence in any amplified product was found. Results of array CGH on the PRSG product of the microdissected esophageal cancer tissue showing both the 11q13 amplification and the 9p21 deletion demonstrated that PRSG is also useful for amplification of a small amount of DNA samples obtained from methanol-fixed, paraffin-embedded tissues (Fig. 4D).

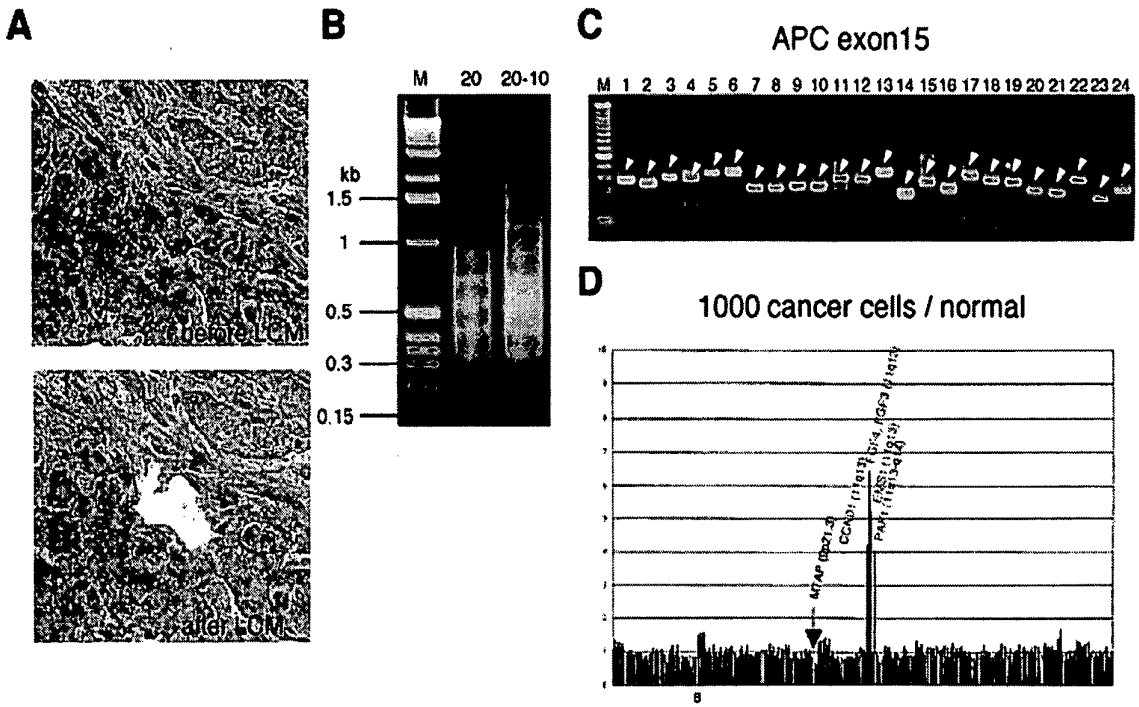

Figure 4. Application of PRSG to DNA samples from methanol-fixed and paraffin-embedded tissues. **A:** Separation of cancer cells from an esophageal cancer tissue by LCM. A section (8 μm) was stained with H & E. Approximately 1,000 esophageal cancer cells were transferred to a transfer film, followed by DNA extraction. **B:** An ethidium bromide–stained agarose gel electrophoretograph of 1 μg of PRSG products, produced by first PCR cycled 20 times (20) and second PCR cycled 10 times (20–10) by use of Ex*Taq* DNA polymerase. **C:** Twenty-four DNA fragments corresponding to exon 15 of *APC*, amplified by PCR from 100 ng of a PRSG product, were analyzed by ethidium bromide–stained 2% agarose gels. Target DNA fragments for PCR, arrowheads. **D:** Results of array CGH on the PRSG product of a microdissected esophageal cancer tissue. Fluorescence ratios on all of the 287 chromosome loci between two PRSG products of both microdissected esophageal cancer cells (~1,000 cancer cells) and a normal DNA are shown. The 11q13 amplified genes (red) or the 9p21 deleted genes (blue) are also indicated. B, no DNA spot on the array used.

DISCUSSION

There is a series of reports by our group and other groups for genome representation procedures, which are based on an adaptor-ligation–mediated PCR by use of a universal primer of restriction enzyme–digested DNA fragments (Ko et al., 1990; Sasaki et al., 1994; Inoue et al., 1996; Lucito et al., 1998; Ohki et al., 1998; Klein et al., 1999; Makrigiorgos et al., 2002). Representations (amplified DNAs) are useful for some types of genetic analyses, including measuring relative gene copy number by Southern blot (Ko et al., 1990; Sasaki et al., 1994; Lucito et al., 1998) or CGH (Klein et al., 1999; Makrigiorgos et al., 2002) and LOH (Inoue et al., 1996; Lucito et al., 1998; Ohki et al., 1998), although only 12–24% of the human genome was captured when we used a single 4-base cutter such as *Mse*I or *Sau*3AI. Some DNA fragments have high GC contents and may form a stable secondary structure, which often prevents PCR amplification at a standard annealing temperature, 50 to 60°C. However, we were able to conduct PCR by use of a specific adaptor acting at a high annealing temperature, 72°C, to minimize biased amplification resulting from differences in template sequences (Inoue et al., 1996; Lucito et al., 1998; Ohki et al., 1998; Klein et al., 1999; Makrigiorgos et al., 2002). We also confirmed an alternative use of an adaptor-ligation–mediated PCR at a high annealing temperature on high-fidelity global mRNA amplification for in vivo gene expression profiling of as few as 100 cells obtained by LCM (Aoyagi et al., 2003). For these reasons, we used an adaptor-ligation–mediated PCR procedure for WGA.

To improve coverage in capturing the genome, we used randomly fragmented DNAs as a PCR template because complete genomic DNA sequences of any organism were obtained by sequencing of subclones, which were prepared by sheared DNAs of BAC clones or the genome DNAs.

Of all of the processes in PRSG, only the hydrodynamic procedure for controlled and unbiased DNA shearing is relatively time-consuming. An automated system, by which multiple samples can be treated at once, should be used. It could be noted that the percentage of captured exons in PRSG products of DNA from formalin-fixed, paraffin-embedded tissues varied from 50 to 70%, possibly because of differences in the use of formalin (data not shown). Nonetheless, these products were useful for array CGH analysis and LOH analysis by use of microsatellites. In our experience in the Millennium Genome Project for high-throughput genetic studies in Japan, at least more than 95% of exons were always retained from both whole blood and methanol-fixed, paraffin-embedded tissues.

In summary, our data suggest that PRSG can provide us with a sufficient amount of genomic sequence for a variety of genetic analyses as well as for long-term storage for future work.

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